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Integrated operation of continuous chromatography and biotransformations for the generic high yield production of fine chemicals

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Abstract

The rapid progress in biocatalysis in the identification and development of enzymes over the last decade has enormously enlarged the chemical reaction space that can be addressed not only in research applications, but also on industrial scale. This enables us to consider even those groups of reactions that are very promising from a synthetic point of view, but suffer from drawbacks on process level, such as an unfavourable position of the reaction equilibrium. Prominent examples stem from the aldolase-catalyzed enantioselective carbon–carbon bond forming reactions, reactions catalyzed by isomerising enzymes, and reactions that are kinetically controlled. On the other hand, continuous chromatography concepts such as the simulating moving bed technology have matured and are increasingly realized on industrial scale for the efficient separation of difficult compound mixtures – including enantiomers – with unprecedented efficiency. We propose that coupling of enzyme reactor and continuous chromatography is a very suitable and potentially generic process concept to address the thermodynamic limitations of a host of promising biotransformations. This way, it should be possible to establish novel in situ product recovery processes of unprecedented efficiency and selectivity that represent a feasible way to recruit novel biocatalysts to the industrial portfolio. © 2006 Elsevier B.V. All rights reserved.

Keywords: Reactive chromatography; Biocatalysis; Simulated moving bed; Aldolases; Isomerases; Process integration

1. Introduction

Biocatalysis has become a workhorse in the chemical industry for the production of pharmaceutical

intermediates (Straathof et al., 2002; Liese et al., 2000). In particular, reactions with enzymes from the classes 1 (redox enzymes) and 3 (hydrolases) have been exploited. The most prominent reasons for this are ease of access to starting materials and enzymes. In particular the latter point has undergone some drastic improvements over the last 15 years due to a variety of developments, such as (i) large genome-scale

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(Fleischmann et al., 1995) and metagenomic sequencing programs (Venter et al., 2004); (ii) the screening of large metagenomic expression libraries (Lorenz and Eck, 2005); (iii) constantly increasing abilities of tailoring enzymatic properties to specific needs (such as improved enantioselectivity or process stability; Reetz, 2001); and (iv) recombinant enzyme production. These facilitate drastically the potential transition of new biocatalytic concepts from the stage of fundamental chemical research to application in industrial processes (Glieder et al., 2003; Greenberg et al., 2004).

With the enzyme sourcing problem reduced, process considerations come more and more into focus, addressing such important questions as suitable cofactor regeneration protocols (Stampfer et al., 2002; Wandrey et al., 2000), or reactions in non-conventional media (Kragl et al., 2002; Klibanov, 2001).

Thus, we can pose now the question whether by smartly coupling biocatalytic and process engineering steps we can design generic process schemes that extend our arsenal of industrial biocatalysis. One such area could be, for example, reactions whose yield is limited by thermodynamic constraints, which might require carrying out biocatalysis and product removal in one unit at the same time. A prominent example is isomerase catalyzed reactions. Usually, in such reactions the operator is left with a potentially difficult to process mixture of starting material(s) and product(s). In addition, in order to achieve decent yields on an expensive starting material (in reactions involving at least two starting materials), the cheaper starting material is often applied in (large) excess, adding to the difficulty of purification. Consequently, such reactions have remained largely unexplored on preparative level. A general process option to increase the yield could recruit entire new groups of enzyme with already proven synthetic merit to the preparative fine chemist's tool-box. This would include such exciting areas as the manufacturing of saccharides and enantioselective formation of C–C bonds, but also more established product groups such as amino acids. In particular enzymes from the classes 4 (lyases) and 5 (isomerases), which are frequently used in these groups of reactions, suffer from an unfavourable position of the reaction equilibrium (Goldberg et al., 2004), but also many hydrolase-catalyzed kinetically controlled synthetic reactions face essentially the same problem (Wegman et al., 2001; van Rantwijk et al., 1999).

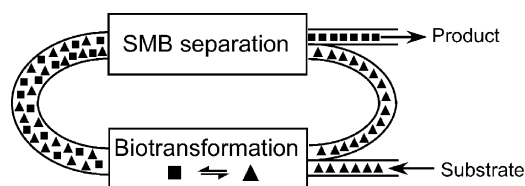


Fig. 1. Concept of an on-line coupling of continuous chromatography and biotransformation.

An obvious solution to the problem is to selectively remove the product immediately or shortly after its formation from the reactor. This can be achieved, for example, by separating the compound mix in a continuous chromatograph, removing the product, and returning the remaining starting material to the reactor (Fig. 1). Depending on the type of reaction, both operations could even be carried out in one reactor (see below). The required continuous chromatography has emerged over the last decade as an excellent and industrially viable process option for the separation of a wide range of product mixtures in two or more fractions (Juza et al., 2000). Chromatography's high resolution power provides also a unique opportunity to accommodate the fact that (bio)catalysts frequently do not change the physicochemical properties of a molecule by much, usually rendering work-up of mixtures difficult. Furthermore, continuous chromatography considerably reduces the eluent consumption and product dilution compared to traditional batch chromatography schemes, making it a much more attractive procedure (Nicoud, 1992; Miller et al., 2003; Grill et al., 2004; Strube et al., 1998a, 1998b).

Obviously, such a coupled operation of a continuous chromatography installation and an enzyme reactor creates a set of constraints for the applicable conditions. This extends at least to the choice of eluent (which is at the same time the reaction medium of the enzyme reactor), which leads to the question of suitable column materials (stationary phases) in the chromatography unit. In this work, we will explore the feasibility of coupling continuous chromatography and enzyme reactors based on previous experiences. We will first discuss possible configurations that combine biotransformation and chromatographic separation and analyze them in terms of for which types of enzyme-catalyzed reactions they might be used. Next, we will discuss the most prominent enzyme classes that suffer from

an unfavourable position of the reaction equilibrium. Finally, the continuous operation of a chromatographic unit requires particularly robust stationary phases (SPs) to be used in the chromatography columns. On the other hand, the material must be suitable to separate the most likely product classes with enzyme compatible eluents (Fig. 1). Thus, we investigate in the subsequent part whether there are materials that comply with both requirements. Based on these three elements, we will give some guidelines on how promising biotransformation processes for specific thermodynamically limited reactions can be realized in the future.

2. Biocatalysis integrated with continuous chromatography

2.1. The principle of continuous chromatography

Continuous chromatography has so far been most successfully realized as simulated moving bed technology (SMB). This technology is best understood as a hypothetical true countercurrent chromatography (TCC) (Fig. 2a). TCC is characterized by the countercurrent movement of a liquid and a solid phase. The two inlet flows (eluent make-up and feed) and the two outlet flows (raffinate and extract) are dividing the process into four distinct zones. Separation takes place in zones II and III, and regeneration of liquid and solid phase takes place in zones IV and I. The flow ratio (ratio of velocities) between liquid and solid phase has to be set such that the less retained compound is moving in direction of the liquid phase and is collected at the raffinate port while the more retained compound spends more time adsorbed on the solid phase and moves in the direction of the solid phase, so that it can be collected at the extract port. The fluid flow velocities in the zones can be controlled by the inlet and outlet volumetric flows.

Obviously, the technical realization of such a system is rather difficult, because of the required continuous movement of the solid phase. Therefore, the concept is realized as a simulated moving bed (SMB). In the scope of this discussion only a brief outline of the most important features is presented. A typical SMB consists of 4–24 columns that are connected in an annular alignment by multi-way valves. The true countercurrent movement of liquid and solid phase is simulated by

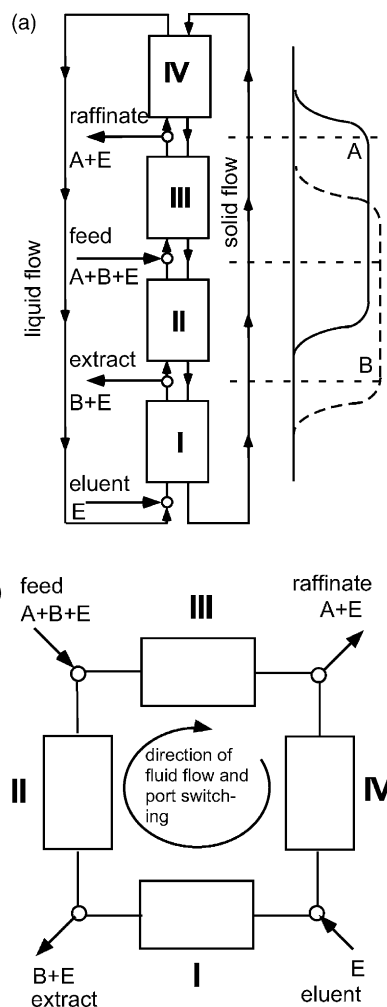


Fig. 2. (a) Schematic diagram of a four-zone true countercurrent chromatography (TCC) unit consisting of four countercurrent chromatography columns with the corresponding axial concentration profile for a binary separation. (b) Schematic diagram of a four zone SMB, the experimental counterpart of a TCC with four columns: (A) less retained compound; (B) more retained compound; (E) eluent.

periodically switching the inlet and outlet flows from column to column in the direction of the fluid flow. Consider for the sake of simplicity a 4-column SMB (Fig. 2b) with one column in each zone and a mixture of species A and B dissolved in the applied eluent entering the SMB between zones II and III at the beginning of a cycle; the weaker retained species A reaches the outlet of zone III first. Before B reaches the end of zone III, the in- and outlets are switched in the direction of

the fluid flow, so the zones are moved by one column in the direction of fluid flow. This translates into a hypothetical movement of the columns (i.e. the SP) in the direction opposite to the fluid flow.

Analogously to TCC, port switching moves the more retained compound from zone III to zone II and eventually to zone I, where complete desorption takes place and pure B can be collected at the extract port between zones I and II. Pure A can be recovered continuously from the raffinate port between zones III and IV, while in zone IV residual A is adsorbed in order to achieve a complete regeneration of the solvent. Possible SMB operating points can be easily derived by application of the triangle theory (Mazzotti et al., 1997), which requires only the adsorption isotherms, which in turn can be obtained easily from HPLC runs.

2.2. Continuous chromatographic bioreactor concepts

When such a continuous chromatography unit is now connected to an enzyme reactor, the potentially improved yield gained by the coupling of the two process steps has to be paid for by a decrease in the degrees of operational freedom as separation and reaction need to be operated under identical process conditions (Fig. 1). There are basically three ways to achieve the coupling of biochemical reactions and continuous chromatography, which vary in the degrees of freedom in operation and design.

2.2.1. Simulated moving bed reactor (SMBR)

The basic operating concept of an SMBR (Fig. 3a) is analogous to that of an SMB. The enzyme is simply added throughout the SMB unit, either to the SP in immobilized form or to the mobile phase in soluble form. Consequently, starting material can be continuously converted in all sections of the SMBR. Consider a thermodynamically limited reaction of type A to B and C. Species A is added to the SMBR in the feed flow and is enzymatically converted to B and C. Species B and C are separated from each other due to their different adsorption behaviour. This separation prevents the backward reaction and enables complete conversion of A. Species B and C can be collected at the extract and raffinate port, respectively, in high purity. Ideally the retention of A should be between that of B and C (Lode et al., 2001). Separation and reaction

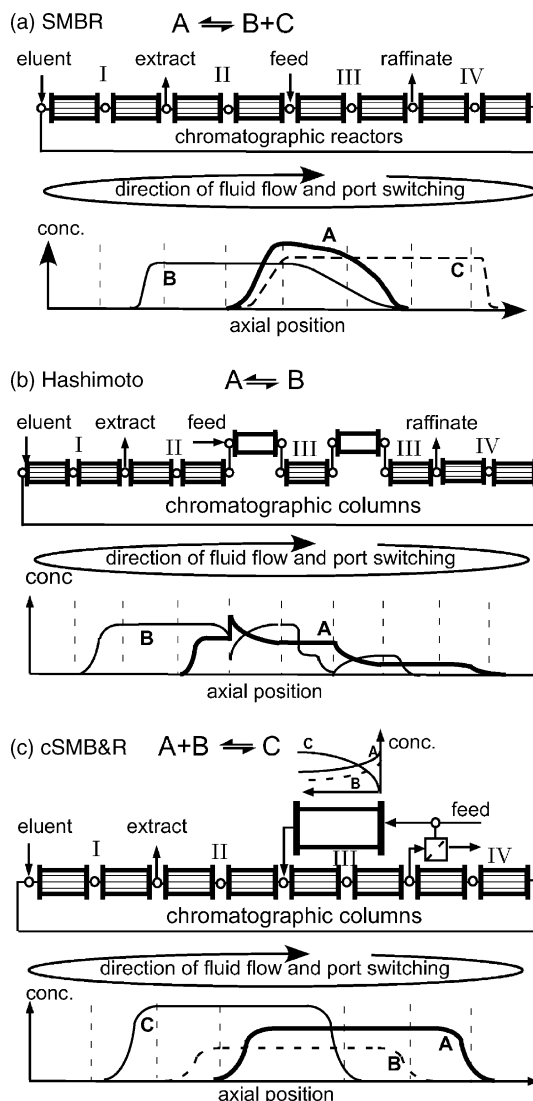


Fig. 3. (a) Simulated moving bed reactor (SMBR) with axial concentration profile of substrate A and products B and C. (b) Hashimoto process with axial concentration profile of substrate A and product B. (c) Coupled SMB and bioreactor (cSMB&R) with axial concentration profile of substrates A and B and product C. Striped rectangles: chromatography columns. Empty rectangles: enzyme reactors.

are not separated in time and space, constituting a high degree of integration. On the other hand, the number of operation points that would be feasible were the SMB operated without reaction is much reduced because reaction kinetics and equilibrium have to be taken into account (Lode et al., 2001). Obtainable productivities at

Table 1
Biotransformation combined with SMB

Educt	Product	Enzyme	Stationary phase	Concept	Eluent	Performance	Remarks	Reference
Sucrose	Glucose fructose	Invertase	CEX, KorelaV70C polystyrene resin (Ca ²⁺ form)	SMBR 12 columns	Water	Complete conversion 16 kg sugar/m ³ SP/day purities >95%	Product inhibition	Akintoye et al. (1991)
Lactose	Galactose glucose	Lactase	CEX Dowex 50WX4 polystyrene resin (Ca ²⁺ form)	SMBR 12 columns	Water	Complete conversion 0.6 kg sugar/m ³ SP/day purities glucose >95%; galactose >77%	Product inhibition	Shieh and Barker (1996)
Sucrose	Dextran fructose	Dextran-sucrase	CEX Purolite PCR563 polystyrene resin (Ca ²⁺ form)	SMBR 12 columns	Water	Complete conversion purities fructose >99%; dextran >92%	Prevention of fructose acting as acceptor	Barker et al. (1992, 1993)
Starch	Maltose dextrin	Maltogenase	CEX KorelaV70C polystyrene resin (Ca ²⁺ form)	SMBR 12 columns	Water	60% conversion >3 kg sugar/m ³ SP/day purities maltose >95%; dextrin >93%	Product inhibition	Shieh and Barker (1995)
Glucose	Fructose	Immobilized glucose isomerase	Y zeolite (Ca ²⁺ form)	Hashimoto 16 chromatographic columns 7 enzyme columns	Water	Purity fructose >52%	Yield beyond equilibrium	Hashimoto et al. (1983)
Propionic acid isoamyl alcohol	Isoamyl propionate water	Immobilized lipozyme IM	CEX Dowex HCR-W2 polystyrene resin (Na ⁺ form)	SMBR 3 columns	Hexane isoamyl-alcohol	26.4 kg ester/kg SP/day	Yield beyond equilibrium/enzyme inactivation	Mensah and Carta (1999)
Propionic acid 2-ethyl-1,3-hexanediol	Ester water	Immobilized lipozyme IM	CEX Dowex HCR-W2 polystyrene resin (Na ⁺ form)	SMBR 5 columns	Hexane 2-ethyl-1,3-hexanediol	~3 kg ester/kg SP/day	Yield beyond equilibrium/enzyme inactivation	Meissner and Carta (2002)
Sucrose lactose	Lactosucrose glucose (byproduct fructose)	β- Fructofuranosidase	CEX Amberlite CR-1310 polystyrene resin (Na ⁺ form)	SMBR 12 columns	0.1 mM NaCl	70% conversion but hydrolysis of product at raffinate port	Yield beyond equilibrium	Kawase et al. (2001)
Sucrose	Glucose fructose	Invertase	CEX Monosphere 99 polystyrene resin (Ca ²⁺ form)	SMBR 12 columns	Buffer pH 4.5 (acetic acid/Ca-acetate)	Complete conversion 170 kg/m ³ SP/day purities >90%	Product inhibition	Azevedo and Rodrigues (2001)

Realized model processes combining SMB and biotransformation. The problems addressed were mostly product inhibition, low equilibrium yield or enzyme inactivation. The process concepts are described in detail in the text. CEX: cation exchanger.

complete separation and complete conversion are generally decreased in comparison to a purely separative SMB (Ströhlein et al., 2005).

The few realized model processes combining biotransformation and SMB technology are almost exclusively based on the SMBR concept (Table 1) with reactions leading to at least two products. In earlier works, the SMBR was investigated as a two zone SMBR without the zones for regenerating eluent and SP (Akintoye et al., 1991; Barker et al., 1992, 1993; Shieh and Barker, 1995, 1996). Regeneration of the SP was accomplished by decoupling the last column of the extract zone (equivalent to zone II of Fig. 3a) and eluting the strongly adsorbed compound with fresh eluent. Regeneration of the eluent was omitted and the complete effluent from the raffinate zone (SMB zone III) was collected (open loop arrangement). The enzymes were dissolved in the mobile phase in all these cases. Rather than thermodynamic limitations, the model reactions addressed product inhibition and reaction selectivity. It was shown that the applied concept required only 34–72% of the amount of enzyme needed in a corresponding conventional batch reactor of the same productivity (Shieh and Barker, 1996). Later, the concept was further developed into a closed-loop four zone SMBR by Azevedo and Rodrigues (2001).

This concept was also used for overcoming thermodynamic limitations in lipase catalyzed esterifications (Mensah et al., 1998). Mensah et al. (1998) and Mensah and Carta (1999) realized a simplified SMBR, consisting of only one zone of several columns, constituting the separation and reaction zone. Two thirds of the SP on a weight basis consisted of immobilized lipase, the rest of ion exchanger material. The alcohol and acid, dissolved in hexane, were fed to the first column, which was periodically removed in order to recover the adsorbed water separately by elution with pure alcohol. The column was then added to the separation and reaction zone at the last position. Later, a three-zone SMBR was applied in an open loop arrangement that omitted only the solvent regeneration zone (Meissner and Carta, 2002). Hexane and the alcohol were used as eluent, whereas the acid was added with the feed. Water accumulation on the immobilized enzyme was reduced by removing the water at the extract port. Both concepts keep water adsorption on the immobilized enzyme below a threshold value and thus prevent product hydrolysis.

2.2.2. The Hashimoto process

In order to prevent reactions from occurring in all SMB zones, a partial disintegration of separation and reaction is required. The enzymatic reaction is then no longer distributed throughout the SMB, but localized in enzyme reactors that are fixed to specific SMB zones (e. g. zone III) while the columns are switched from zone to zone (Hashimoto et al., 1983) (Fig. 3b). The advantage of such an alignment in comparison to an SMBR can easily be understood from a thermodynamically limited reaction of type A to B. The weaker retained species A moves with the mobile phase and is stepwise converted to species B in the reactors present in zone III. This process concept creates the possibility to install reactors only in those SMB zones, where reaction is advantageous. In zones where enriched product is present (zones I and II), enzyme reactors would only promote the backward reaction counteracting the conversion and resolution achieved before. Theoretically, the fraction of not converted A could be collected at the raffinate port and recycled to the feed flow in order to increase the product yield (Borren and Schmidt-Traub, 2004).

Experimentally, this concept was used for the production of high fructose syrup. The enrichment of fructose beyond equilibrium was established by the stepwise conversion of glucose in the bioreactors present in zone III while the more retained fructose could be collected at the extract port. Since in the realized process alignment no regeneration of the mobile phase was implemented (no zone IV), considerable amounts of residual glucose could reach the fructose outlet and thus limit the achievable purities (Hashimoto et al., 1983).

2.2.3. On-line coupling of SMB and reactor (cSMB&R)

In this concept, SMB and bioreactor constitute two separate units interconnected by a loop. The SMB continuously separates product and starting material leaving the bioreactor. Considering for example a thermodynamically limited biochemical reaction of types A to B, the starting material A is recycled from one port of the SMB unit to the bioreactor while the product B can be collected at the other effluent flow ideally completely separated from A. Thus theoretically 100% yield can be achieved. In SMB operation, generally dilution of the compounds to be separated occurs with respect to the feed concentrations. Since the effluent

flows are usually higher than the feed flow only a fraction of the volume of the effluent can be recycled for continuous operation. This needs to be compensated for by e.g. nanofiltration resulting in a concentration of the residual starting material before recycling. In cSMB&R reaction and separation are decoupled as far as possible, establishing a broad range of possible applications.

Although the cSMB&R concept has – to the best knowledge of the authors – never been experimentally realized, various authors have compared the potential performance of the cSMB&R concept by model-based simulations to other process concepts. For the glucose/fructose isomerisation, the Hashimoto process was superior compared to a cSMB&R process with respect to eluent consumption for fructose contents in the range of 45–65% (Hashimoto et al., 1983). At higher fructose purities application of the cSMB&R concept would prove advantageous (Hashimoto et al., 1983). This result was extended by later work, where the SMBR, a cSMB&R–Hashimoto hybrid and a cSMB&R process were compared, again for glucose/fructose isomerisation (Borren and Schmidt-Traub, 2004). The SMBR process appeared to be the most advantageous for fructose purities smaller than 65%, the cSMB&R–Hashimoto hybrid process for purities between 65 and 95%, and the cSMB&R for purities >95%. The fact that highest purities can be obtained from the cSMB&R configuration, can be easily explained by the fact that in SMBR for equilibrium reactions of types A to B the backward reaction is constantly catalyzed (the enzyme being present throughout the reactor). This way, high productivity at high purities is prevented. Due to the continuous recycling of residual glucose in the cSMB&R process, the best performance in terms of productivity can be obtained at very high purities.

In summary, it becomes clear that coupled continuous reaction and chromatographic separation systems have addressed mostly food-related biotransformations that suffered from product inhibition and so far have not played a major role for fine-chemical purposes, and in particular have not addressed thermodynamically limited reactions in considerable depth. On the other hand, even the rather limited set of examples from various application areas already indicates that such integrated process concepts represent unique options to drive reactions to complete conversion by realizing

a highly selective in situ product recovery. Their potential for producing products at high purities and decent productivities makes them particularly attractive for the fine chemical field with pharmaceutical applications in mind.

Next, we will investigate the scope of the opportunity with respect to the interesting catalysts that would benefit from a combined enzyme reactor/SMB operation.

3. Accessible enzyme classes

As the structure of the catalyzed reactions (one or more products) has obviously an impact on the most advantageous configuration of the coupled enzyme reactor/SMB, it appears useful to arrange the type of enzyme reactions according to number of reactants involved:

3.1. Reactions of types A to B

The easiest reactions that can be considered for integrated operation with a continuous chromatography installation are those that convert a starting material A into a product B, i.e. isomerases, epimerases, or racemases (Table 2). Most of the reactions catalyzed by this group have starting materials and products of very similar Gibbs' energy of formation, resulting in equilibrium positions that leave mixtures of substrate and product. The most prominent model system here is the conversion of glucose to fructose by a D-xylose isomerase (see above) to produce high fructose corn syrup (Häusler and Stütz, 2001). From a fine chemical point of view, this reaction might not be the most interesting one, but it serves well to illustrate the potential of the concept for another set of isomerase-catalyzed reactions that open access to interesting ketoses via the corresponding aldoses whose chemistry is much better developed (Häusler and Stütz, 2001). Here, quite a range of interesting isomerases is available, including D-xylose isomerases for the synthesis of iminoalditol-based glycosidase inhibitors (Häusler and Stütz, 2001), L-fucose and L-rhamnose isomerase for the production of rare sugars such as L-lyxose (Granström et al., 2005), and L-arabinose isomerase to produce D-tagatose as a potential bulk sweetener (Kim et al., 2003).

Table 2
Accessible enzyme classes in combination with SMB

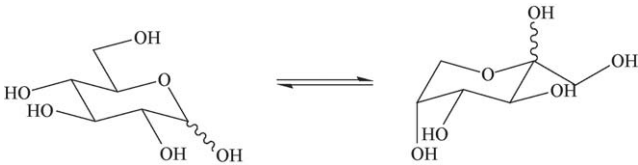


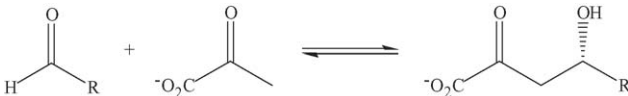
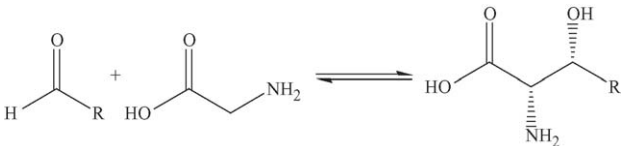
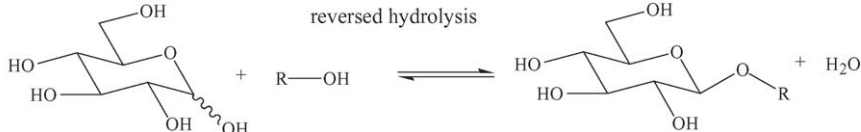
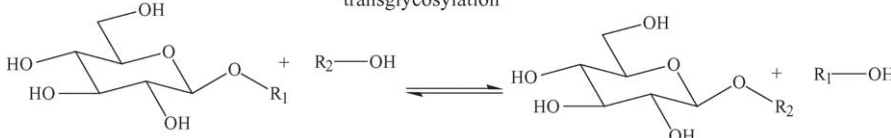
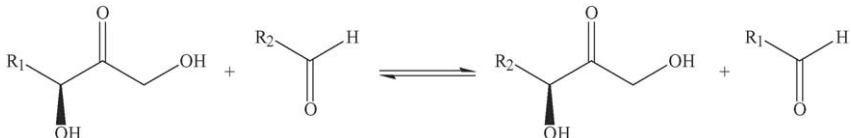

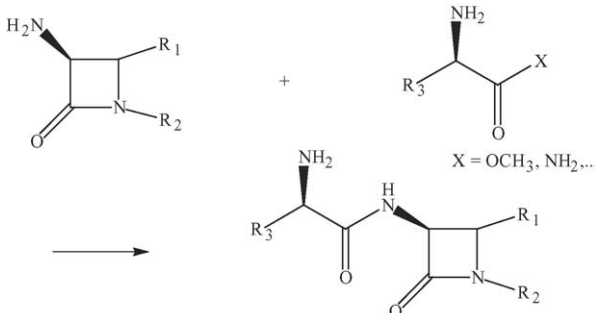
Enzyme (example)	Reaction	EC number	Requirements
Isomerases: D-xylose-isomerase		5.3.1.5	Mn ²⁺ , Mg ²⁺ , Co ²⁺
Epimerases: D-tagatose-3-epimerase		5.1.3.X	None
Racemases: amino acid racemase		5.1.1.10	PLP
Pyruvate-dependent aldolases: N-acetylneuraminic aldolase		4.1.3.3	None
Glycine-dependent aldolase: L-threonine aldolase		4.1.2.5	PLP

Table 2 (Continued)

Enzyme (example)	Reaction	EC number	Requirements
	 <p>reversed hydrolysis</p>		
Glycosidases: β-glucosidase	 <p>transglycosylation</p>	3.2.1.X	Mg ²⁺ , Na ⁺
Transketolase		2.2.1.1	Mg ²⁺ , TPP
Transaminases: L-aspartate aminotransferase		2.6.1.X	PLP
Kinetically controlled reactions: penicillin acylase	 <p>X = OCH₃, NH₂, ...</p>	3.5.1.11	None

Overview of potentially interesting enzyme reactions coupled to SMB (PLP: pyridoxal phosphate; TPP: thiamine diphosphate).

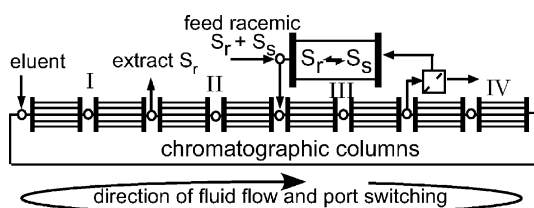


Fig. 4. Process scheme for the production of enantiopure compounds by the integrated operation of chiral SMB and a racemase reactor. S_R : *R*-enantiomer. S_S : *S*-enantiomer.

The epimerases represent another interesting set of enzymes that interconvert either different aldoses or different ketoses into each other. In particular D-tagatose-3-epimerase offers access to a number of rare D-sugars e.g. D-sorbose (Itoh et al., 1995) and the potential bulk sweetener D-psicose (Takeshita et al., 2000).

Racemases (Table 2) catalyze the interconversion of enantiomers and are therefore potentially of particular interest in fine chemistry. In the context of integrated processes, they might allow a novel perspective on the relationship between the number of directly accessible enantiomers and the number of required enzymes. For example, the classic resolution routes to amino acids usually start from racemic amino acid intermediates that are then enantioselectively hydrolyzed. Direct access to either one of the enantiomers with 50% yield requires two enzymes, one for each enantiomer. Getting to 100% yield requires a third enzyme, the racemase (or a suitable alternative chemical procedure). Using a combination of a racemase and a continuous chromatography system, only one enzyme would be required to access both enantiomers in a theoretical yield and enantiomeric excess of 100% from the same installation (Fig. 4).

Racemases have already found their way into industrial catalysis (Schnell et al., 2003; May et al., 2002) in order to remove some of the 50% yield limitation in classical resolution processes of activated amino acid intermediates. A number of racemases that operate on amino acids directly (rather than their derivatives) have been reported (Schnell et al., 2003), indicating that this product group might be as well accessed directly by integrated operations rather than via activated intermediates. Other efforts to develop racemases for biotransformations have mainly focused on racemases acting on α -hydroxy carbonyl derivatives such as mandelic acid (Schnell et al., 2003).

3.2. Reactions of types $A + B$ to C

Lyases are important in fine chemistry due to their capacity of enantioselective C–C bond formation. In the group of aldolases, a typically essential donor substrate reacts with one of a variety of possible acceptor aldehydes. Consequently, aldolases are organized along the essential donor into dihydroxyacetonephosphate-(DHAP), acetaldehyde-, pyruvate-, or glycine-dependent aldolases. The latter two aldolase classes (Table 2) catalyze many highly interesting reactions with unfavourable reaction equilibria. One important example here is the conversion of pyruvate and *N*-acetyl-D-mannosamine (ManNAc) to *N*-acetylneuraminic acid by the pyruvate-dependent *N*-acetylneuraminic aldolase. This reaction is of interest for the production of, for example, neuraminidase inhibitors such as Relenza (Glaxo Smith Kline). As the manufacturing of ManNAc from *N*-acetylglucosamine is also challenging, only relatively dilute substrate mixtures are applied and the product is quite heavily contaminated with substrates ($K_{eq} = 28.7 \text{ M}^{-1}$ at 25°C) (Kragl et al., 1991). A number of approaches have been followed to address the problem, all involving an excess of pyruvate to drive the reaction (Blayer et al., 1999; Kragl et al., 1991; Mahmoudian et al., 1997). As this behaviour is a general property of the pyruvate-dependent aldolases for the production of 2-ketoaldonic acids, the removal of pyruvate as an end-of-pipe treatment received considerable attention (Mahmoudian et al., 1997; Wong, 1998). This example illustrates clearly the underlying problems of this specific enzyme class.

The same problem is encountered in the manufacturing of β -hydroxy-amino acids with glycine-dependent aldolases (Table 2). Here, threonine aldolases represent a synthetically interesting group of enzymes because they have the potential for selective production of diastereomerically complete sets of β -hydroxy amino acids (theoretically consisting of four enzymes for all four stereochemically possible permutations of the C_α and C_β atom of an amino acid). Although the enzyme set is not (yet) complete (Liu et al., 2000), the industrial potential for these enzyme is already clear (Liu et al., 1999; Kreuzman et al., 1997).

Furthermore, there is strong evidence that glycine-dependent aldolases that are reported as indifferent to the configuration at C_β are in fact diastereose-

elective to a considerable extent, but the diastereomeric excess suffers from extended reaction times because the kinetically preferred isomer has time to be converted until the diastereomerically more unfavourable thermodynamic equilibrium has been reached (Kimura et al., 1997) (see also below—note that this behaviour does not make these enzymes of the $A + B$ to $C + D$ type, because for the reverse reaction the enzyme requires only C or D , not both). We will address this behaviour in the next paragraph in more detail.

3.3. Reactions of types $A + B$ to $C + D (+E)$

This type contains mostly hydrolase reactions, for many of which it would be interesting to reverse hydrolysis, as it is done in lipase catalyzed synthesis in organic media. One prime example for a reaction that requires an aqueous medium and would therefore potentially benefit a lot from the enzyme reactor/SMB concept is the alkylation of glycosides with glycosidases. Although these enzymes catalyze *in vivo* the hydrolysis of glycosides, they are heavily explored as biocatalysts in the synthetic direction as well, either in reverse hydrolysis or in transglycosylation (van Rantwijk et al., 1999). For reverse hydrolysis, by adding alcohols as glycosyl acceptors to the aqueous reaction medium and thus reducing the water activity in the reaction mixture considerably ($a_w \leq 0.4$), promising yields up to >60% (for the acceptor) can be obtained (Vic and Crout, 1995; Chahid et al., 1994) which make this a potentially highly attractive class of reactions for integrated processing.

Transketolases catalyze *in vivo* the reversible transfer of a two-carbon ketol unit from a ketose to an aldose. Synthetically, they open the door to the manufacturing of commercially interesting enantiomerically pure diols and triols with *D-threo*-stereochemistry (Turner, 2000) and have attracted a lot of interest because a promising way to overcome the problem of equilibrium position is available: by using hydroxypyruvic acid (HPA) as ketol donor, CO_2 is produced and this drags the reaction theoretically to completion (Chauhan et al., 1997). However, HPA is expensive, and the ability to substitute this ketol donor by one of a host of others that are accepted by transketolases might very much broaden the interest in this class of enzymes (Bolte et al., 1987).

A similar strategy has been applied for transaminases that can be used to produce for example unnatural amino acids from the corresponding α -keto acids (Ager et al., 2001; Fotheringham, 2000). Using L-aspartate as the amino donor, oxaloacetate remains which spontaneously decarboxylates to pyruvate and thus prevents the reverse reaction. Again, removing the requirement for a particular amino-donor might broaden the utility of this class of enzymes. However, in both of the above examples, a one-(transketolases) or two-(transaminases) compound mix at the end of the reaction would need to be exchanged for a complex four-compound-mixture that would need to be suitably separated by the continuous chromatographic system.

In a number of interesting biocatalytic reactions of this class, the composition of the product mixture varies over time, i.e. first a kinetically controlled product is formed before after longer residence times in the reactor the product mixture approaches the thermodynamically most favourable composition. In fact, this has already been briefly discussed for threonine aldolases (see above). However, kinetically controlled syntheses play also an important role with hydrolases that are used in synthetic direction. In transglycosylation, a disaccharide (or any other “activated” glycoside) is used as a glycosyl-donor in the synthesis of substituted glycosides. This allows to accelerate the reaction considerably (relatively to the above discussed reverse hydrolysis), but bears the problem that water can act as a nucleophile in the enzymatic reaction as well and can cause the donor or the product to hydrolyze, thus creating an undesired dead-end product (van Rantwijk et al., 1999). The same strategy is followed in the manufacturing of semisynthetic antibiotics, where an activated side-chain is coupled to the β -lactam-nucleus using a penicillin acylase. Again, either the activated side chain or the product might be hydrolyzed in the presence of the enzyme (Wegman et al., 2001). Obviously, a strategy that limits the time of exposure of the reactants to the enzyme and allows the selective removal of the “sensitive” product from the mixture should increase the yield (calculated based on either the activated donor or the desired nucleophile).

In summary, there is a large variety of enzymes available that have already proven their merit in terms of chemical utility but face intrinsic processing problems because of the position of the corresponding reaction's equilibrium. Consequently, it appears that there

is ample motivation to investigate process concepts to exploit their synthetic power. Conceptually, they fall in two classes: reactions that lead to only one product and reactions that lead at least to two products. In terms of product, most examples address sugars and sugar-like compounds, and amino acids and their derivatives. The required separations cover separations of entirely different molecules, of stereoisomers, or of enantiomers. Consequently, a large variety of product groups would need to be separated on the SMB. Next, we will analyze whether the SP materials that are currently available are likely to be sufficient to address these diverse tasks.

4. Stationary phases

Due to the integrated operation of enzyme reactor and chromatographic separation unit, possible SPs have to comply with a number of requirements: if an organic solvent has to be added to the eluent, its fraction should be low enough to permit efficient enzyme function, and eluent pH should be adjusted to close to neutral conditions including buffering when necessary. Another point is that enzyme reactions will require buffering or at least some salt addition to adjust the pH properly. This can have important effects on the separation characteristics of the SP. Consequently, we need to investigate the different materials with respect to their behaviour in eluents with a high water content and (close-to) physiological pH.

4.1. Potential stationary phases for coupled enzyme reactor/SMB operation

4.1.1. Non-chiral stationary phases

Non-chiral SPs can be applied for most of the potential applications of the coupled enzyme reactor/SMB concept, because the separation task does not involve the separation of enantiomers from each other. For these separations, reversed-phase silica is the most widely used packing materials in preparative chromatography (Majors, 2004). In its important C₁₈- and C₈-form, it is mostly applied for preparative separations of weakly basic or polar compounds, but cannot tolerate pure aqueous eluents as the alkyl chains bonded to the silica core would collapse. However, this is no issue as long as the enzyme used for biotransformation tolerates a minor content of organic solvents, which

is frequently the case. On the same note, the slightly acidic conditions that are preferred to counter the alkaline susceptibility of the material are typically not a problem for biotransformation.

Ion-exchange materials are commonly used in the analysis of amino acids and are also good SPs for preparative applications. The group of Yonemoto separated glutamic acid and valine by continuous chromatography (Kitakawa et al., 1995) and also a ternary mixture of glutamic acid, valine, and leucine (Fukumura et al., 2000) on a cation-exchange resin in the Na⁺ form using sodium acetate buffer (pH 5) as eluent. Wu and co-workers separated a mixture of the amino acids tryptophan and phenylalanine in SMB using PVP resin (poly-4-vinylpyridine cross-linked) and water (Wu et al., 1998).

Also for the separation of saccharides, ion-exchange is among the most suitable techniques for preparative chromatography due to size exclusion and ligand-exchange effects (Welstein and Sauer, 1984). Strong acid-type polystyrene/divinylbenzene-based cation-exchangers in Ca²⁺ form were already used in continuous chromatography for the separation of fructose, glucose and sucrose in water with 0.5 g/l CaCl₂ (Howard et al., 1988), of fructose and dextran in water (Coelho et al., 2002), and of glucose and fructose in an SMBR in a calcium acetate buffer at pH 4.5 (Azevedo and Rodrigues, 2001). Zeolites are an attractive alternative for the separation of sugars as was demonstrated for glucose and fructose with Ca²⁺ exchanged Y zeolites in an SMB with water as eluent (Ching et al., 1987; Hashimoto et al., 1983).

4.1.2. Chiral stationary phases

When the enzyme reactor/SMB concept is applied to racemases, enantiomers need to be separated from each other. Given the importance of chirality in pharmaceutical chemistry, it is obvious that this is an important potential application. However, there has been no integrated operation of enzyme reactor and chiral SMB before, so there is hardly any information about the behaviour of chiral SPs under enzyme-compatible conditions.

Polysaccharide SPs are considered the workhorse for chiral separation on both analytical and preparative scale due to the broad range of applicable molecules (Zhang et al., 2005) and an outstanding saturation capacity of up to 100 mg/g chiral SP (Francotte, 2001).

Table 3

Overview of potentially suitable preparative stationary phases for a potential integration with enzyme reactions

Stationary phase	Accessible compounds	Applicable enzyme compatible eluents	Potential accessible enzyme reactions
Reversed phase silicas (C18)	Water soluble, non-polar or weak polar	Aqueous buffer, 10% organic modifier required	L-Aspartate aminotransferase, penicillin acylase, N-acetylneuraminic aldolase, transketolase
Ion-exchanger (organic polymers, zeolites)	Charged molecules, sugars	Aqueous buffer	β -Glucosidase, D-xylose isomerase, D-tagatose-3-epimerase, L-threonine aldolase, L-aspartate aminotransferase, transketolase
Macrocyclic glycopeptides ^a	Amino acids, carboxylic acids, β -lactams	Aqueous buffer usually containing >20% organic modifier	Amino acid racemase

^a Only preparative chiral stationary phase yet applied with high water systems.

They can be operated in reverse mode with water contents up to 90% (manufacturer's information), however sufficient interaction with the SP requires neutral analytes as solvation of ionic analytes presumably prevents proper interaction with the SP. Hence, for the separation of basic and acidic compounds pH adjustment or addition of a counterion for ion pair formation is required. Furthermore the interactions in chiral discrimination of polysaccharides SPs are considered to be less efficient in highly polar aqueous eluents such as water (Tachibana and Ohnishi, 2001). Other preparatively applicable chiral SPs include tartardiamide chiral SPs, SPs with low molecular-mass chiral selector (i.e. Brush type, Pirkle type) and polyacrylamide chiral SPs. Those SPs show saturation capacities of approximately 1–20 mg/g CSP and are stable in reverse and normal phase, but are usually operated in normal phase mode (Francotte, 2001; Andersson and Allenmark, 2002).

Another broadly applicable class of preparative chiral SPs that received considerable attention over the last years is macrocyclic glycopeptide bonded phases that are reported to show good separation characteristics for a variety of compounds, in particular amino acids, when employing aqueous eluents (Xiao and Armstrong, 2003). For example, baseline resolution of racemic methionine was obtained on an analytical teicoplanin-based SP column employing pure water as solvent (Berthod et al., 1996; Bechtold et al., 2006).

The different types of chiral SPs are complementary and frequently mutually exclusive in their separation characteristics. A screening of 55 different enantiomeric pairs employing different polysaccharide and macrocyclic glycopeptide chiral SPs revealed that –

except for two – all enantiomeric pairs could be separated with five compounds being only separated on the macrocyclic glycopeptide chiral SPs and 17 only on the polysaccharide chiral SPs (Anderson et al., 2003).

Next to these fundamental considerations, selection criteria for the best SP include separation efficiency, mechanical stability at elevated pressure and chemical stability (Juza et al., 2000). However little specific information is documented for these issues.

In summary, it is clear that sufficient materials are available to select SPs for coupled enzyme reactor/SMB operation for the most interesting product classes (Table 3). Of course, this very general statement has to be verified in each specific case, because it is based on some rule of thumb assumptions about enzyme behaviour in the presence of low amounts of organic solvents.

5. Guidelines

In the sections above, we have analyzed the available reactor configurations for coupled enzyme reactor/SMB operation, the chemical opportunities that could be addressed by such a concept, and in how far proper SP material is available so that novel processes might indeed be realized. We will now try to accommodate the separate points into some design guidelines.

5.1. Choice of process configuration

The Hashimoto process requires the most complex process control of the three investigated configurations

due to additional equipment such as reactors, valves, etc. Furthermore, the purities that can be obtained for thermodynamically limited reactions are generally lower than in SMBR and cSMB&R configuration due to the cascade-like arrangement of reactors and separators. Recycling concepts (e.g. Hashimoto–cSMB&R hybrids) for residual substrate might increase the product yields for the Hashimoto process. However the additional equipment expenses and design complexity will only in very exceptional cases justify an application in coupled enzyme reactor/SMB operation.

For the remaining two concepts – SMBR and cSMB&R – selection depends on the applied type of reaction. The SMBR concept is most advantageous for reactions forming two different molecules, e.g. the A and B to C and D, or A to B and C type, that is for reverse hydrolysis of glycosides, transketolase- and transaminase-catalyzed reactions. Due to the rather long residence times in the SMBR combined with the instantaneous separation of substrate(s) and products, complete conversion can usually be achieved for thermodynamically limited reactions. At the same time, the products are separated to high purities at decent productivities. When applying the SMBR concept to reactions that lead to only one product, the permanent back-reaction throughout the whole unit interferes with high product purity, so the concept is less suitable. Similarly, in batch reactive chromatography (e.g. a single column equipped with both catalyst and adsorbent) this would translate to a considerable contamination of the product elution profile with substrate. Furthermore, there is considerable loss of enzyme if this is added to the eluent.

On the other hand reactions resulting in the formation of only a single product, e.g. A to B, or A and B to C are preferably carried out in a cSMB&R. This covers aldolases and isomerases, epimerases, and racemases. Here the product can be rapidly removed from the enzyme reactor and the starting material is recycled to the enzyme reactor. This leads to high purities, theoretically 100% yield, and short residence times in the enzyme reactor, making this a suitable configuration for kinetically controlled reactions. However as dilution of the compounds is common in SMB operation – especially for the extract – an additional concentration device for the starting material might be necessary adding to the process costs and complexity. Neverthe-

less, there is strong motivation to explore this configuration.

5.2. Choice of stationary phase

The choice of the appropriate SP mostly depends of the product class to separate. Considering the enzymes shown in Table 2, most products will have sugar-like or amino acid-like characteristics. This narrows down selection of materials to ion-exchangers or zeolites for sugars and sugar-like compounds and adsorbents or ion-exchangers for amino acids (Table 3). A wide range of these materials have already been applied successfully in preparative applications as described above.

Furthermore, enzyme reactions commonly take place in buffered systems and sometimes require the presence of cofactors (Table 2). Simple salts are mostly used as buffer, whereas cofactors might vary from divalent cations like Mg^{2+} to larger molecules like pyridoxal phosphate (PLP). The presence of these compounds may strongly influence separation characteristics especially if ionic interactions are governing the retention of the compounds to be resolved. This, for example, excludes the use of anion-exchangers for enzymes requiring PLP as cofactor, as PLP carries a double negative charge and would therefore be strongly retained on anion exchanger.

Still, given the many materials that are available to realize the different separation tasks, it appears likely that coupled enzyme reactor/SMB will be a very versatile concept to address a broad variety of different reactions. However, many of the potential materials have not been tested under enzyme compatible conditions yet, so care has to be taken here.

5.3. Economics

SMB units require high investments but on the other hand offer a high degree of automation. Hence, with increasing scale the plant costs' contribution to the total costs generally decreases while stationary and mobile phase costs dominate. Strube and co-workers have calculated the cost distribution for SMB separation of fructose and glucose (100% purity) on a 20,000–200,000 t/a scale and the production of an enantiopure drug on a 1–5 t/a scale. The costs for the non-chiral SP employed in the sugar separation are negligible, while about two-thirds of the total costs are generated by solvent costs

(water in this case). For chiral separations, the costs for the SP make a considerable contribution to the total costs but still solvent costs are the major cost driver (Strube et al., 1998a). Desirable productivities for chiral SMB as rule of thumb are 0.3 kg enantiomer per kg CSP per day (Nicoud, 1999) and reported SMB applications with chiral polysaccharide SPs realize a productivity of 0.5–1.5 kg/kg CSP/day (Francotte, 2001).

Such productivities can be translated roughly into separation prices per kilogram of product that have to be added specifically to the enzyme catalysis step. For the production of enantiopure naproxen on 100 t/a scale by SMB separation of the racemic mixture, the separation costs were calculated to be up to 31 \$/kg assuming a productivity of 1 kg/kg CSP/day. With increasing productivity the manufacturing costs decrease drastically e.g. via 10 \$/kg at 2 kg/kg CSP/day down to 3 \$/kg at 5 kg/kg CSP/day (Bruno, 2004). With the ongoing improvements made in SMB operation (e.g. Varicol: Ludemann-Hombourger et al., 2000; Power-Feed: Zhang et al., 2003; ModiCon: Schramm et al., 2003) as well as in the development of new powerful SPs such productivities should be achievable and thus costs reduced.

6. Conclusions

The advances in biotechnology allow us to consider a whole new range of enzymes for industrial-scale fine chemistry. Some of these novel opportunities – such as those enzymes that catalyze thermodynamically limited reactions – require novel processing concepts. Realizing such concepts by integrating continuous chromatography as SMB and enzyme reaction appears to be a very attractive option – the SMB concept is sufficiently mature, financially attractive, and flexible for this purpose, and enzyme-compatible stationary phases/eluent systems are likely to be found for many of the potential biocatalytic opportunities. We suggest that there will be many applications of this technology in the near future.

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